**SMARCB1/INI1 Tumor Suppressor Gene Is Frequently Inactivated in Epithelioid Sarcomas**

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**Abstract**

Epithelioid sarcoma is a rare soft tissue neoplasm of uncertain lineage that usually arises in the distal extremities of adults, presents a high rate of recurrences and metastases and frequently poses diagnostic dilemmas. The recently reported large-cell “proximal-type” variant is characterized by increased aggressiveness, deep location, preferential occurrence in proximal/axial regions of older patients, and rhabdoid features. Previous cytogenetic studies indicated that the most frequent alterations associated with this tumor entity affect chromosome 22. In this study, combined spectral karyotyping, fluorescence in situ hybridization, and array-based comparative genomic hybridization analyses of two proximal-type cases harboring a rearrangement involving 10q26 and 22q11 revealed that the 22q11 breakpoints were located in a 150-kb region containing the **SMARCB1/INI1** gene, and that homozygous deletion of the gene was present in the tumor tissue. The **SMARCB1/INI1** gene encodes for an invariant subunit of SWI/SNF chromatin remodeling complex and has been previously reported to act as a tumor suppressor gene frequently inactivated in infantile malignant rhabdoid tumors. We analyzed **SMARCB1/INI1** gene status in nine additional epithelioid sarcoma cases (four proximal types and five conventional types) and altogether we identified deletions of **SMARCB1/INI1** gene in 5 of 11 cases, all proximal types. We confirmed and further extended the number of cases with **SMARCB1/INI1** inactivation to 6 of 11 cases, by real-time quantitative PCR analysis of mRNA expression and by **SMARCB1/INI1** immunohistochemistry. Overall, these results point to **SMARCB1/INI1** gene involvement in the genesis and/or progression of epithelioid sarcomas. Analysis of larger series of epithelioid sarcomas will be necessary to highlight putative clinically relevant features related to **SMARCB1/INI1** inactivation. (Cancer Res 2005; 65(10): 4012-9)

**Introduction**

Epithelioid sarcoma is a rare and distinctive soft tissue sarcoma first described in 1970 (1). This neoplasm typically arises in the distal extremities of young adults as a painless, slow-growing nodule within dermis or subcutis or, less commonly, in deep fascial or tenosynovial tissue. Morphologically, the classic, also called “distal”, form of epithelioid sarcoma consists of tumor cells that range from spindle to large round or polygonal, bearing a strong similarity to epithelioid or squamous cells, and arranged in nodular aggregates that commonly exhibit central necrosis. Upon microscopic examination, tumor cells show slight nuclear atypia, vesicular nuclei, and small nucleoli (2–7). Two variants of this form have been described: the “angiomatoid,” characterized by pseudoangiosarcomatous features and the “fibroma like,” with a predominance of spindle cell pattern with minimal cellular pleomorphism. The immunophenotype of epithelioid sarcoma shows positivity for vimentin and both epithelial markers EMA and cytokeratin. A number of the cases are also positive for CD34, muscle-specific and smooth muscle actins, neuron-specific enolase, and S100 protein (8–12).

Recently, an atypical large cells variant of epithelioid sarcoma, which is characterized by increased aggressiveness, multinodular pattern of growth, rhabdoid features and deep location, has been described (13–15). It mainly but not exclusively occurs in the pelvic, perineal and genital tract as well as in the proximal extremities of middle aged adults and therefore it has also been referred as “proximal-type” epithelioid sarcoma (PES). Upon microscopic examination, PES consists of large epithelioid carcinoma-like and/or rhabdoid cells. Despite the differences in clinical presentation and behavior, conventional epithelioid sarcoma and proximal-type epithelioid sarcoma share a similar immunophenotypic profile suggesting a common pathogenesis. It is not clear yet if the unfortunate behavior of PES is related to the prominent rhabdoid phenotype or merely to classic prognostic factors, such as tumor size, depth, proximal/axial location, resectability, or vascular invasion (2, 13, 14).

Conventional cytogenetic studies of epithelioid sarcoma revealed complex karyotypes with various chromosome deletions and gains, but the only recurrent breakpoints in structural rearrangements were 18q11 and 22q11 in two cases each (16–25). Spectral karyotyping (SKY) analysis of six PES revealed the presence of 11 rearrangements on chromosome 22q in five of the six cases analyzed, with two cases showing a similar t(10, 22) translocation (26). In addition, loss of heterozygosity analysis identified frequent deletion of 22q material in epithelioid sarcoma (27). These data suggest that a region on chromosome 22q may contain a tumor suppressor gene involved in the development of this neoplasm.

In this study, 11 epithelioid sarcomas, six proximal types and five conventional types, were analyzed by cytogenetic and molecular approaches. We provide evidence of genomic deletion of the **SMARCB1/INI1** gene in 5 of 11 (45%) epithelioid sarcoma cases that correlates with mRNA and/or protein down-regulation. **SMARCB1/INI1** immunohistochemistry confirmed inactivation at the protein level and extended the number of cases with **SMARCB1/INI1** alterations to 6 of 11 (55%), of which 5 of 6 are proximal types. Our data suggest the involvement of **SMARCB1/INI1** alterations in a substantial fraction of epithelioid sarcoma.

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

P. Modena and E. Lualdi contributed equally to this work.

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Materials and Methods

Patients and pathology. This study included 11 epithelioid sarcoma cases. Ten were retrieved from the files of the Department of Pathology, Istituto Nazionale per lo Studio e la Cura dei Tumori, Milano, Italy, comprising the period 1991 to 2002. One additional case was collected at the Portuguese Oncology Institute, Porto, Portugal. In all cases, routine sections were available. Six cases showed features consistent with PES, 3 with rhabdoid-like and 2 with epithelioid-like appearance. In one case (case 5), the tumor exhibited a major spindle-cell component admixed with epithelioid-like features. Five cases corresponded to classic epithelioid sarcoma, distal type. Immunophenotyping was in keeping with the morphologic diagnosis in all the 11 cases, showing positivity for low-weight cytokeratin, epithelial membrane antigen, and vimentin. CD34 yielded positive results in cases 4, 6, 10, and 11. S100 protein staining was positive in case 2. Patients’ clinical data, including gender, age, tumor location, and histologic type and subtype are summarized in Table 1.

Cell lines. Previously described (26) short-term tumor-derived cell cultures of cases 1 to 4 were cultured with Amniomax C100 (Life Technologies, Carlsbad, CA) supplemented with Amniomax C100 supplement (Life Technologies) and harvested with 0.01 g/mL Colcemid (Karyomyx Colcemid, Life Technologies) overnight. Cell lines 293 and G-401 were purchased from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Life Technologies).

Fluorescence in situ hybridization analysis. BAC probes used for dual-color fluorescence in situ hybridization (FISH) were extracted using QIAPrep Midiprep (Qiagen, Valencia, CA), verified by PCR amplification of sequence-tagged site markers and labeled with Spectrum Green dUTP (Vysis, Inc., Downers Grove, IL) by Nick Translation sequence-tagged site markers and labeled with Spectrum Orange dUTP and G-401 were purchased from American Type Culture Collection (Manassas, VA) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (Life Technologies).

Table 1. Clinical data and tumor phenotype of the 11 epithelioid sarcoma cases under study

<table>
<thead>
<tr>
<th>Case</th>
<th>Gender/age</th>
<th>Tumor location</th>
<th>Morphology</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>F/31</td>
<td>thigh</td>
<td>Proximal ES, epithelioid like</td>
</tr>
<tr>
<td>2</td>
<td>F/47</td>
<td>perineum</td>
<td>Proximal ES, rhabdoid like</td>
</tr>
<tr>
<td>3</td>
<td>M/71</td>
<td>dorsal region</td>
<td>Proximal ES, rhabdoid like</td>
</tr>
<tr>
<td>4</td>
<td>M/30</td>
<td>lumbar region</td>
<td>Proximal ES, epithelioid like</td>
</tr>
<tr>
<td>5</td>
<td>M/36</td>
<td>sacral region</td>
<td>Proximal ES, prominent spindle-cell component</td>
</tr>
<tr>
<td>6</td>
<td>F/66</td>
<td>inguinal region</td>
<td>Proximal ES, rhabdoid like</td>
</tr>
<tr>
<td>7</td>
<td>M/27</td>
<td>arm</td>
<td>Distal ES, classic type</td>
</tr>
<tr>
<td>8</td>
<td>F/55</td>
<td>foot</td>
<td>Distal ES, classic type</td>
</tr>
<tr>
<td>9</td>
<td>M/31</td>
<td>hand</td>
<td>Distal ES, classic type</td>
</tr>
<tr>
<td>10</td>
<td>M/22</td>
<td>hand</td>
<td>Distal ES, classic type</td>
</tr>
<tr>
<td>11</td>
<td>M/27</td>
<td>arm</td>
<td>Distal ES, classic type</td>
</tr>
</tbody>
</table>

Abbreviation: ES, epithelioid sarcoma.
were analyzed using ABI PRISM Sequence Detection System software (version 2.1, Applied Biosystems). The use of normal muscle or normal kidney cDNAs as calibrator provided comparable results (data not shown).

**Mutation analysis.** Mutation detection of SMARCB1/INI1 gene was done by exons amplification and direct sequencing using previously described primers (29).

**Western blot analysis.** Western blot analysis was done using standard techniques from total lysates extracted from frozen tumor tissue and cell lines. Frozen tissue was preliminarily homogenized using Mixer Mill MM300 (Qiagen). From each sample, 40 μg of proteins were separated on 10% SDS-PAGE and electro-blotted onto polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, United Kingdom). Subsequently, membranes were incubated 1 hour at room temperature in a solution of PBS supplemented with 5% nonfat dry milk. For immunodetection, the anti-BAF47/SNF5 antibody (BD Transduction Laboratories, Franklin Lakes, NJ) was used. After overnight incubation at 4°C with the primary antibody diluted 1:250, membranes were washed in TBST [10 mmol/L Tris-HCl (pH 8.0), 0.15 mol/L NaCl, and 0.05% Tween 20], followed by horseradish peroxidase–conjugated goat-antimouse or goat-antirabbit antibody. Enhanced chemiluminescence (Amersham Biosciences) was used for detection. Protein loading equivalence was assessed using an anti-β-actin antibody (Sigma).

**Immunohistochemistry.** Immunohistochemistry analysis was done by autostainer instrument together with ChemMate detection kit 5001 (Dako, Milano, Italy) using heat-induced epitope retrieval. Cytokeratin staining required trypsin predigestion. Antibodies used were low-weight Cytokeratin (mouse monoclonal, 1:10, BD Transduction Laboratories), EMA (mouse monoclonal, 1:200, Dako), CD34 (mouse monoclonal, 1:10, BD Transduction Laboratories), Vimentin (mouse monoclonal, 1:50, Dako), BAF47/SNF5 (BD Transduction Laboratories, Franklin Lakes, NJ), and is flanking clone RP11-71G19 that showed three signals indicating that it spans the breakpoint (Fig. 1B), whereas overlapping clones RP11-61P17 and RP11-80O7 showed signals only on the der(10) and der(22), respectively (Fig. 1C). Intriguingly, BAC clone RP11-71G19 contains SMARCB1/INI1 gene, a known tumor suppressor gene inactivated in malignant rhabdoid tumors (30, 31). FISH experiments in case 2, that harbored a complex rearrangement involving chromosomes 10, 15, and 22 (Fig. 1D), showed the loss of clone RP11-80O7 that overlaps with RP11-71G19 and is flanking $SMARCB1/INI1$ gene, therefore suggesting a similar breakpoint region as in case 1 (Fig. 1E–F).

With the same strategy in case 3, carrying a t(12;22), the breakpoint on chromosome 22 was found to be located in a 1-Mb region of 22q12 (Fig. 1F). $EWSR1$ gene did not seem rearranged as shown by FISH and Southern blot (data not shown); furthermore, results.

**Results**

**Fluorescence in situ hybridization analysis of chromosome 22 breakpoints in short-term tumor cell cultures.** In our previous report of SKY analysis in six PES cases (26), we described a cluster of breakpoints on 22q11 chromosome band and the presence of a similar t(10;22) translocation in two cases. To map the chromosome 22 breakpoints more precisely, we performed FISH experiments using a panel of BAC clones in four of the six cases previously analyzed by SKY. Notably, the breakpoints in cases 1 and 2 were found located within a 150-kb region covered by the BAC clone RP11-71G19 and flanked by RP11-61P17 and RP11-80O7, that overlap 5'- and 3'-ends of RP11-71G19, respectively. In case 1 (Fig. 1A), FISH experiments showed that the t(10;22) was balanced, with the exception of clone RP11-71G19, that showed three signals indicating that it spans the breakpoint (Fig. 1B), whereas overlapping clones RP11-61P17 and RP11-80O7 showed signals only on the der(10) and der(22), respectively (Fig. 1C). Intriguingly, BAC clone RP11-71G19 contains SMARCB1/INI1 gene, a known tumor suppressor gene inactivated in malignant rhabdoid tumors (30, 31). FISH experiments in case 2, that harbored a complex rearrangement involving chromosomes 10, 15, and 22 (Fig. 1D), showed the loss of clone RP11-80O7 that overlaps with RP11-71G19 and is flanking $SMARCB1/INI1$ gene, therefore suggesting a similar breakpoint region as in case 1 (Fig. 1E–F).

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**Figure 1.** SKY and FISH analyses of metaphase chromosomes from tumor-derived short-term cell cultures in cases 1 and 2. A and D, visualization of normal chromosomes 22 and aberrant chromosomes involving 22 material after SKY analysis in cases 1 and 2, respectively. For each aberration the RGB (red-green-blue), the inverted DAPI, and the pseudocolor images of the chromosomes (left to right). Numbers on the right side of the derivative chromosomes denote the chromosomal origin. Complete SKY results have been previously published (26). B, FISH analysis of clone RP11 71G19 containing $SMARCB1/INI1$ gene in case 1 displayed splitting of the signal on both derivatives of the translocation t(10;22)(q26;q11). C, double-color FISH analysis of clones RP11-61P17 (green) and RP11-80O7 (red) flanking $SMARCB1/INI1$ gene in case 1 displayed separated signal on der(22) and der(10), respectively. E, the same double-color FISH analysis in case 2 displayed loss of RP11-80O7 (red signal) on der(10). F, summary of FISH results in cases 1 to 4.
the presence of t(12;22)-associated fusion genes EWSRI-DDIT3 and EWSRI-ATF1, typical of myxoid-round cell liposarcoma and clear cell sarcoma, respectively, was excluded by reverse transcription-PCR experiments (data not shown). In case 4, because of the complexity of the rearrangements involving chromosome 22 and insufficient cell pellet, it has only been possible to show the loss of 22q12 and that the breakpoints did not encompass the region covered by RP11-71G19 (Fig. 1F).

We showed the in vivo occurrence of t(10;22) and 22q11 breakpoint in case 1, by double-color interphase FISH experiments from frozen tumor biopsy (Supplementary Fig. A). Several subclones of tumor cells were present, in which either both derivatives were retained (shown by split signals) or one/two derivatives were lost (absence of signal).

Array-based comparative genomic hybridization and fluorescence in situ hybridization analysis of tumor tissue samples 1 and 2. Whole genome array-CGH analysis of cases 1 and 2 at 2- to 4-Mb resolution displayed a normal chromosome 22 content in case 2 and a 22q heterozygous loss covering ~15 Mb and comprising SMARCB1/INI1 in case 1 (Fig. 2A-B). Because in malignant rhabdoid tumors SMARCB1/INI1 gene is homozygously deleted in a markedly high proportion of the cases (29), and 22q rearrangements frequently seem to trigger SMARCB1/INI1 loss (32), we did interphase FISH with clone RP11-71G19, that contains SMARCB1/INI1 gene, to directly evaluate the SMARCB1/INI1 gene dosage in tumor samples 1 and 2. In case 1, double-color FISH analysis on touch preparations of frozen tissue showed a high frequency of clone RP11-71G19 homozygous loss and control clone RP11-91K24 heterozygous loss (Fig. 2C). These two clones are separated by 3 Mb, and therefore SMARCB1/INI1 homozygous loss lies within the wide region of heterozygous loss detected by array-CGH but is below the resolution of the array employed. Similarly, case 2 displayed no evidence of loss by array-CGH, but double-color FISH analysis on paraffin-embedded tumor tissue showed attainment of normal copy number of clone RP11-91K24 and homozygous loss of clone RP11-71G19 (Fig. 2D), suggesting that the region of loss comprising SMARCB1/INI1 is below the resolution of the array employed.

Additional double-color interphase FISH experiments were done to define the extent of the regions of homozygous deletion (Supplementary Fig. B). In case 1, the results showed homozygous deletion of clone RP11-71G19 in the majority of nuclei that retained the signal of the flanking clones RP11-61PI17 and RP11-8007. Thus, the combined array-CGH and FISH results allowed us to narrow down the region of homozygous loss to less then 150-kb and to strongly pinpoint SMARCB1/INI1 as the gene inactivated by the observed rearrangements. Two additional known genes are present in this region, pre-B lymphocyte gene 3 (VPREB3) involved in B-cell differentiation and matrix metalloproteinase 11 (MMP11), a gene reported overexpressed in a variety of tumors (33–35). In case 2, double-color FISH experiments were done on paraffin sections and, together with array-CGH and FISH results reported in Fig. 2B and D, indicated that the extent of the region of HD is between 450 kb and 5 Mb (Supplementary Fig. B).

Whereas molecular analyses (either array-CGH or FISH) of PES tumor biopsies 1 and 2 disclosed a loss of SMARCB1/INI1 gene, cytogenetic and FISH analyses of short-term cell culture of the same cases had revealed chromosome 22 rearrangements in the vicinity of SMARCB1/INI1 gene but no evidence of loss. Such a supposed discrepancy between results obtained on short-term cell culture and frozen biopsy suggested both that a specific tumor cell clone was selected during in vivo cell culture and that the translocation involving 22q preceded loss of 22q11 material. These hypotheses are substantiated by FISH results reported in Supplementary Figs. A and B.

Fluorescence in situ hybridization analysis of SMARCB1/INI1 locus in paraffin-embedded tumor samples. We extended the analysis of SMARCB1/INI1 locus to the 11 epithelioid sarcoma cases under study (Table 1) by double-color interphase FISH with clone RP11-71G19 that contains SMARCB1/INI1 gene, in combination with a control probe from 22q13 (RP11-262A13) on paraffin-embedded tumor tissue. With the exception of case 3, which did not display any loss by FISH, all other PES cases (cases 1, 2, 4-6) revealed intratumor loss of SMARCB1/INI1 signal (Fig. 3, b and β). Interestingly, it seemed evident that SMARCB1/INI1 loss did not correlate with the rhabdoid cell compartment. In fact, case 1 morphology was homogeneously epithelioid, carcinoma like (Fig. 3, 1a), and displayed a high proportion of homozygous loss (Fig. 3, 1b). Case 2 had a multinodular pattern with rhabdoid cells...
and showed homozygous loss (Fig. 3, 2a and b); case 4 displayed SMARCB1/INI1 loss confined to a region of epithelioid morphology (Fig. 3, 4a and b). Interestingly, in case 5, SMARCB1/INI1 homozygous loss was restricted to areas of spindle cell morphology whereas epithelioid-like areas were normal (Fig. 3, 5a-b and 5a-b). Finally, case 6, of prominent rhabdoid morphology, displayed homogeneous heterozygous loss (Fig. 3, 6a and b). Cases 7-11 displayed a normal SMARCB1/INI1 copy number by FISH and case 11 is shown as a representative example in Fig. 3, 11a and b.

**SMARCB1/INI1 protein expression analysis.** To circumvent the potential limitations related to the presence of normal cells infiltrating the tumor and to further investigate SMARCB1/INI1 inactivation at the protein level, immunohistochemistry was done using a monoclonal antibody against SMARCB1/INI1 protein.

**Table 1.**

<table>
<thead>
<tr>
<th>Clone signals</th>
<th>RP11-71G19 (SMARCB1) 0 1 2 3 4</th>
<th>RP11-262A13 0 1 2 3</th>
<th>αBAF47</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1</td>
<td>161 19 20</td>
<td>85 112 3</td>
<td>-</td>
</tr>
<tr>
<td>Case 2</td>
<td>200</td>
<td>78 120 2</td>
<td>-</td>
</tr>
<tr>
<td>Case 3</td>
<td>1 11 35 53 50</td>
<td>66 81 1</td>
<td>+</td>
</tr>
<tr>
<td>Case 4</td>
<td>82 63 5</td>
<td>2 65 83</td>
<td>-</td>
</tr>
<tr>
<td>Case 5a</td>
<td>65 84 1</td>
<td>2 66 82</td>
<td>+</td>
</tr>
<tr>
<td>Case 5c</td>
<td>99 16 35</td>
<td>3 68 78 1</td>
<td>-</td>
</tr>
<tr>
<td>Case 6</td>
<td>84 110 6</td>
<td>1 82 115 2</td>
<td>-</td>
</tr>
<tr>
<td>Case 7</td>
<td>1 86 112 1</td>
<td>2 87 111 + + + + +</td>
<td></td>
</tr>
<tr>
<td>Case 8</td>
<td>2 3 144 1</td>
<td>3 7 140</td>
<td>+</td>
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<tr>
<td>Case 9</td>
<td>1 92 104 3</td>
<td>75 122 3</td>
<td>-</td>
</tr>
<tr>
<td>Case 10</td>
<td>89 110 1</td>
<td>3 86 111 + + + + +</td>
<td></td>
</tr>
<tr>
<td>Case 11</td>
<td>90 106 4</td>
<td>91 108 1 + + + + +</td>
<td></td>
</tr>
</tbody>
</table>
Immunohistochemistry showed absence of the protein in 6 of 11 (55%) cases, including 5 of 6 PES samples and 1 of 5 conventional epithelioid sarcoma samples. One additional conventional epithelioid sarcoma (case 7) displayed focal expression. In total, all (5 of 5) cases displaying SMARCB1/INI1 loss at the molecular level also displayed absence of the protein, and 5 of 6 cases without apparent gene alterations retained complete or focal protein expression (Fig. 3). Control normal tissues as well as internal controls (derma, lymphocytes) displayed nuclear staining. No significant correlation was present between protein expression and age, sex, primary tumor location, and survival. Representative examples of SMARCB1/INI1 immunohistochemistry results are reported in Fig. 3, c.

In selected cases for which sufficient material was available, whole cell lysates were prepared from frozen tissue and analyzed by Western blot using a monoclonal antibody against SMARCB1/INI1 (Fig. 4, f). Lysates from normal kidney cell line 293 and mouse brain were used as positive controls, whereas cell line G-401, derived from a malignant rhabdoid tumor of the kidney, was used as negative control. Two protein isoforms were visible of ~47 kDa, likely corresponding to the two previously described SMARCB1/INI1 splicing variants that differ for 27 bp in exon 2. Concordant with immunohistochemistry results, minimal protein expression was suggested in cases 1, 5, and 9, compatible with the presence of normal cells infiltrating the tumor, whereas protein expression was retained in cases 3 and 8. In case 7, reduced protein expression, consistent with the focal pattern observed by immunohistochemistry, was also suggested.

Molecular analysis of SMARCB1/INI1 locus. To better elucidate the mechanism of SMARCB1/INI1 inactivation in epithelioid sarcoma, we analyzed the gene status in more detail in each case where frozen tissue was available (cases 1-3 and 5-11).

Real-time quantitative reverse transcriptase-PCR analysis of SMARCB1/INI1 mRNA expression revealed that the transcript level was down-regulated in four samples (cases 1, 2, 6, and 9) that displayed loss of SMARCB1/INI1 protein expression, compared with samples that retained at least focal protein expression by immunohistochemistry (Fig. 4B). The mRNA down-regulation was not evident in sample 5 that displayed loss of protein expression in the spindle-cell component of the tumor, either because of the confounding effect of the tumor component that retained SMARCB1/INI1 expression, or because of the presence of post-transcriptional mechanisms of inactivation. Similarly, case 7 that displayed focal protein expression by immunohistochemistry retained a SMARCB1/INI1 transcript level comparable to SMARCB1/INI1-positive tumor samples.

Southern blot analysis using a complete coding sequence SMARCB1/INI1 cDNA as probe revealed an underrepresentation of...
the gene, compared with an EWSR1 control probe located on the same chromosome, in cases 1, 2, 5, and 6, and absence of abnormal fragments (data not shown), suggesting that in cases 1 and 2 the previously described chromosome 22 breakpoints likely did not interrupt SMARCB1/INI1 gene.

Semiquantitative PCR-based DNA analysis of exon 1 of SMARCB1/INI1 gene on the same samples provided concordant results, displaying evidence of gene loss in four of nine samples (cases 1, 2, 5, and 6; Fig. 4C). Donor-derived genomic DNA served as positive control, whereas genomic DNA from G-401 cell line (carrying a homozygous deletion of the entire SMARCB1/INI1 gene) was used as negative control.

To circumvent the limitations due to the presence of normal cells infiltrating the tumor, in some cases with distinct morphologies the same paraffin-embedded sections analyzed by FISH were dissected to isolate normal tissue areas as well as tumor areas with different morphology (Fig. 4D). DNA was extracted and PCR amplified for SMARCB1/INI1 as well as control primer sets. Case 1 confirmed the presence of loss of the entire gene in epithelioid, carcinoma-like areas. Case 5 displayed homozygous loss of exon 1 in areas of spindle cell morphology and case 6, which carried a homozygous heterogeneous loss of SMARCB1/INI1 locus by FISH (Fig. 3, 6b), displayed suggestive indication of complete loss of exon 4 in areas of rhabdoid morphology.

All epithelioid sarcoma cases were analyzed for mutations by exons amplification and direct sequencing, and no mutations were found. Therefore, despite the reduced or absent protein expression, no genetic alterations were identified in cases 7 and 9, respectively. In case 9, however, the down-regulation of SMARCB1/INI1 mRNA was clearly shown by real-time PCR (Fig. 4B). Similarly, ~10% to 20% of malignant rhabdoid tumors are reported not to display any mutation of SMARCB1/INI1 exons. It will be of interest to analyze in detail these cases, to search for alternative mutational events that have not been examined in the present report, such as epigenetic silencing, intronic or promoter mutations, or RNA editing.

Discussion

SMARCB1/INI1 protein was first identified as an interactor protein of HIV Integrase (36) and is supposed to facilitate both the provirus integration into transcriptionally active regions of the host genome and the virus assembly release (37). Additionally, in the recent years, SMARCB1/INI1 gene has been defined as a tumor suppressor gene involved in the development of a rare, aggressive pediatric cancer termed malignant rhabdoid tumor (MRT), that is in fact characterized by biallelic inactivation of SMARCB1/INI1 gene (30, 31). Peculiarly, the spectrum of SMARCB1/INI1 somatic mutations includes a high rate, ~40% of the cases, of homozygous deletions, that are frequently associated with chromosomal translocations and other rearrangements of 22q11 (29, 32, 38). Furthermore, constitutional mutations have also been described causing the highly penetrant hereditary tumor-predisposing syndrome termed “rhabdoid predisposition syndrome” associated with renal and extrarenal MRT and brain tumors (39).

Recent experimental evidence showed that SMARCB1/INI1 protein constitutes an invariant subunit of SWI/SNF-related chromatin remodeling complexes, 2-MDa complexes that regulate transcription by modulating nucleosomal structures in an ATP-dependent manner. Several observations linked the SWI/SNF-related complexes to the pathogenesis of human malignances, including the direct interaction of single components of SWI/SNF complex with pRB and BRCA1 (40–42) and the increased tumor incidence in heterozygous mouse mutants for subunit BRG1 (43).

Functional characterization of SMARCB1/INI1 provided insights into its involvement in tumorigenesis: reintroduction of SMARCB1/INI1 gene in MRT cell lines blocked cell proliferation, induced cellular senescence via CDKN2A/p16/ pRb pathway and modulated actin cytoskeleton organization (44, 45). Furthermore, it was shown that SMARCB1/INI1 protein directly interacts with MYC and is necessary for MYC-mediated transactivation through SWI/SNF complex (46). Interaction of SMARCB1/INI1 with both ENU and AF10, two fusion partners of acute leukemia-associated HRX/MLL protein, as well as with GADD45, a DNA damage-inducible factor whose function is repressed by HRX/MLL fusion proteins, has been reported (47, 48). Therefore, SMARCB1/INI1 tumor suppressor gene is involved in key biochemical pathways implicated in cell growth and development. Additional evidence of the tumor suppressor role of SMARCB1/INI1 was provided by the recently reported mouse models (49–52). Despite the fact that SMARCB1/INI1 knocked out (KO) mice are not vital, both heterozygous and conditional KO mice develop tumors, including MRTs of the central nervous system, lymphomas and, notably, undifferentiated soft tissue tumors.

The identification of inactivating mutations of SMARCB1/INI1 gene in the vast majority of both renal and extrarenal MRTs and the absence of mutations detected in many other pediatric cancers (53), provided strong evidence of the existence of this pediatric neoplasm as a distinctive entity. Nonetheless, it is widely accepted that the histologic hallmark of MRTs (i.e., the rhabdoid features) still represents a nonspecific morphology in rare cases of other pediatric as well as adult neoplasms (13, 54). The identification of SMARCB1/INI1 inactivation in epithelioid sarcoma extends the spectrum of tumors harboring SMARCB1/INI1 mutations to a morphologically peculiar sarcoma of young adults. Based on the present results it could be argued that epithelioid sarcoma might represent an additional entity among the extrarenal MRT spectrum. However, several lines of evidence, in addition to the differences in clinical features, distinguish epithelioid sarcoma from MRT: (i) the gamut of morphologic features presenting in epithelioid sarcoma, which encompasses epithelioid, rhabdoid and spindle cell growth, in contrast to the homogeneous rhabdoid growth pattern shown by MRT; (ii) the spectrum of SMARCB1/INI1 mutations thus far identified in epithelioid sarcoma, which seems restricted to deletions and not to include the point mutations frequently observed in MRT. Unexpectedly, these alterations seem to affect various morphologic tumor components rather then to be confined to rhabdoid morphology; (iii) the heterogeneous distribution of the deletions observed thus far in epithelioid sarcoma, which suggests that, contrary to MRT, at least in some cases they represent relevant secondary tumor–associated changes and not the primary event causing the tumor onset.

Analysis of large, unselected series of epithelioid sarcomas will be necessary to provide further insights into the relationship between SMARCB1/INI1 mutations and clinicopathologic features.

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